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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : C12N 5/02, 11/10, A61K 37/02 C12N 1/38

A1

(11) International Publication Number: WO 89/ 06686

(43) International Publication Date: 27 July 1989 (27.07.89)

(21) International Application Number: PCT/US89/00033

(22) International Filing Date: 9 January 1989 (09.01.89)

(31) Priority Application Number: 144,890

(32) Priority Date: 13 January 1988 (13.01.88)

(33) Priority Country: US

(60) Parent Application or Grant
(63) Related by Continuation
US 144,890 (CON)
Filed on 13 January 1988 (13.01.88)

(71) Applicant *(for all designated States except US):* THE UPJOHN COMPANY [US/US]; 301 Henrietta Street, Kalamazoo, MI 49001 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only) :* FISCHER, H., David [US/US]; 5804 East HJ Avenue, Kalamazoo, MI 49004 (US). BABCOCK, Merrill, S. [US/US]; 5350 Azalea, Portage, MI 49002 (US).

(74) Agent: NEWTSON, Ruth, Hattan; Patent Law Department, The Upjohn Company, Kalamazoo, MI 49001 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), US.

Published
With international search report.

(54) Title: ENHANCED PRODUCTION OF CELLULAR PROTEINS USING BUTYRATE

(57) Abstract

The invention teaches a process to enhance the efficiency of specific protein production by cultured eukaryotic cells by adding an effective amount of butyric acid or salt thereof to the culture growth medium.

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ENHANCED PRODUCTION OF CELLULAR PROTEINS USING BUTYRATE

Field of Invention

The invention teaches a process to enhance the efficiency of specific protein production by cultured eukaryotic cells by adding an effective amount of butyric acid or salt thereof to the culture growth medium.

Background of the Invention

Economical production of rare proteins from cultured cells requires processes which maximize productivity of specific gene products from expression systems compatible with large scale production. In non-secretory or expression systems involving lytic infections, the cells themselves are consumed in the total process of synthesizing and harvesting the products. Expression systems that secrete the desired proteins are preferred because the cells act as true catalysts converting medium components into more complex and valuable biochemicals. Secretory systems also provide the option of isolating products independently from the producing cells, which themselves can be retained within the bioreactor for further production.

The total amount of protein produced by a secretory expression system is modelled by the equation $P = kCVt$, where k represents the specific productivity of the cultured cells in units of protein produced per cell in a unit of time, C represents the density of biocatalytic cells in the bioreactor expressed as number of cells per unit volume, V represents the bioreactor volume, t represents the time of the production period, and P represents the total number of protein units produced. Altering one or more of the variables in this equation provides opportunities for increasing the total amount of protein produced in a bioreactor. For example, either the total culture time or the culture volume can be increased and lead directly to increased amounts of total production. Similarly, higher cell density is achieved by manipulating medium composition and environmental factors. The basic productive unit in the expression system is the producing cell itself.

Processes to increase protein production by increasing the total number of cells and/or the integrated time during which the cells are productive are known in the art. However, effective methods to manipulate the specific productivity of each cell are not available.

Improvements in individual cell productivity are important because total production increases as well as overall efficiency of production. Improved per cell productivity also magnifies any improvements in the other production variables. In addition, improved cell productivity can be exploited to minimize the culture volume or duration reducing capital and labor costs for any given quantity of product. Finally, increased productivity per cell provides higher initial product titers and specific activities which simplifies downstream purification processes and lowers this cost.

Recombinant DNA technologies provide opportunities for higher productivity of proteins for each cell. For example, the construction of chimeric genes with the coding sequence for a protein under the transcriptional regulation of a heterologous promoter stronger than that which normally precedes the gene in its native environment gives higher protein production. In another approach, the DNA sequence coding for the desired protein has been molecularly spliced into a larger DNA construct which can be amplified within a host cell to obtain multiple productive copies of the desired gene sequence in any given cell. Unfortunately, recombinant DNA technologies are generally not practical for every product and are limited by the ability to clone DNA sequences coding for the desired protein. Even state-of-the-art production techniques for monoclonal antibodies still depend upon culture of the original hybridoma line producing the desired antibody and when genes are cloned into an appropriate expression vector, expression is still governed by complex mechanisms of differential gene transcription which allow eukaryotic cells precise regulation of gene expression in development, differentiation and metabolism. Therefore, it is desirable that protein production processes have ways to differentially enhance the expression of genes coding for desired protein products relative to the expression of genes coding for the natural background of cellular protein.

Butyric acid can produce reversible morphological and biochemical changes when contacted with cultured cells. Cell specific changes include: induction of integrated viruses; increased antiviral efficiency of interferon; reversion of characteristics of transformed cells to normal biochemical and morphological patterns; modifications in cytoskeletal assembly; and induction of proteins, enzymes, and hormones. These changes may occur because butyric acid

or salts thereof in growth medium induces inhibition of histone deacetylases, modifies histone-DNA associations, alters chromatin structure and affects specific changes in differential expression of genes located in the histone altered regions. The enhancement of protein expression in specific cells, however, is not fully understood.

In addition, most, but not all, treated cells show retarded or arrested cell proliferation while maintaining approximately normal levels of overall protein synthesis. The combination of effects that dissociate cell growth and protein expression, and enhance specific protein production, provide a way to increase cell productivity for a desired protein product. This depends on the ability to specifically apply and direct the pleiotropic effects of butyric acid toward a specific application. However, previously described effects of the compound are highly cell and protein specific, often are contradictory and fail to provide a means to predict the effects of butyric acid.

Moreover, inhibition of cell growth by butyric acid presents its own limitations to use in a production process. In many culture systems it is difficult to harvest spent, product-containing medium from a bioreactor without also simultaneously removing significant numbers of the producing cells themselves. This is most true in cultures comprising free, single-cell suspensions. In such systems, new cell division is required to regenerate the cell numbers lost during the product harvest. If cell growth is arrested the net effect on overall productivity would be negative with time if the enhanced expressions per cell of desired protein did not compensate for the loss of catalytic cells themselves. Thus, to fully exploit the effects of butyric acid on cell productivity, it may be necessary to use a culture system wherein product can be harvested while the cells are retained in the bioreactor.

Immobilization of cells offers advantages to a process for producing rare biochemicals. Immobilization facilitates cell viability, product expression and product isolation by affecting factors such as cell density, cell microenvironment and separation requirements. Immobilized cells can be retained in the culture vessel allowing product synthesis and harvest to be effectively unlinked from cell growth. Immobilization is conveniently achieved on larger scales

with some type of carrier, which itself is more readily separated from the growth medium than single cells themselves by physical means. Appropriate carriers are frequently of two types: The simplest are microcarriers, to which cells, dependent upon or capable of monolayer growth, can attach and form a dense layer of cells on the bead surface. Cells which normally grow in free, single-cell suspension are more difficult to immobilize. These cells must depend on immobilization for use as true catalysts in order to separated them from product and retain them for reuse in a bioreactor. These cells are conveniently immobilized within a second type of carrier, a biocompatible gel or porous bead.

Biocompatible hydrogels can be made from alginate, agarose, polyacrylamide, etc., in which cells can be embedded during bead formation in ways which preserve cell viability and potential for growth. Alternatively, cells can be trapped by, migrate into, and proliferate within sponge-like porous beads. Both types provide benefits of shear protection and high-cell-density microenvironments. Porous gel beads also allow diffusion of secreted proteins from entrapped cells into the surrounding medium and these secreted proteins can be separated from cells by removing the medium. Furthermore, since entrapped cells frequently require less serum levels in the medium to maintain growth than do cells in free suspension, improved final product purity and simplified final product purification methods are possible.

Immobilization is beneficial only as long as the entrapped cells maintain viability and do not leach from the gel into the surrounding medium. It is known, however, that continued proliferation of immobilized cells causes leaching of the cell population from the carrier into the surrounding medium, negating the effects of immobilization and threatening the structural integrity of the beads themselves. One approach to mitigate leaching encapsulates the carrier beads within a semi-permeable molecular membrane. This method suffers in several ways. First, even though the product itself is secreted from the cells, it is retained within the encapsulating membrane, which must be ruptured prior to harvest. This limits use of encapsulated cells to batch harvest systems and precludes the use of continuous perfusion culture protocols required to maximize the use of the cells catalytically. Secondly, the product is retained for long periods

within the capsule at high concentrations with cell and medium derived proteases which threaten product quality and integrity. Therefore, once immobilized cells reach a density sufficient for economical production further proliferation should be inhibited without adversely affecting cell viability, productivity, product integrity or other benefits attained by immobilization.

Approaches previously used to limit growth of immobilized cells are inadequate because these methods to modulate cell growth typically involve limitation of an essential nutrient. Limitation of glucose, glutamine, oxygen or serum have been used to slow cellular proliferation. Low temperature has also been used. These methods all impose stress on the cells and result in sub-optimal metabolism. An improvement in the use of immobilized eukaryotic cells to specifically control growth of immobilized cells under conditions which are fully compatible with cell growth, viability, and productivity is provided by this invention.

Information Disclosure

Several reviews describe general techniques for immobilizing cells: Birmbaum, S., et al., Immobilized Cells, Chapter 15, Solid Phase Biochemistry, 679-763 (1983); Lim, F., Microencapsulation of Living Cells and Tissues 1983 Review and Update, Applied Biochemistry and Biotechnology, 81-85 (1984); Corcoran, E., The Production and Use of Immobilized Living Microbial Cells, Chapter 2, Methods of Immobilizing Living Cells, 12-50 (1984); Chang, T.M.S., Biomedical Applications of Artificial Cells Containing Immobilized Enzymes, Proteins, Cells and Other Biologically Active Materials, Chapter 11, Enzymes and Immobilized Cells in Biotechnology, 263-281 (1985); and Nilsson, K., Methods for Immobilizing Animal Cells, Trends in Biotechnology, 73-78 (1987).

Techniques used to entrap cells in alginate gels are reported in literature and patent documents. The PCT application PCT/US85/00838 teaches the use of permeable gels to aid culturing biological tissue and producing biomolecules. The International Search Report published with the above document lists the following documents as particularly relevant: U.S. Patent 4,407,957; U.S. Patent 4,352,883; U.S. Patent 4,409,331; U.S. Patent 4,452,892; U.S. Patent 4,399,219; U.S. Patent 4,401,755 and U.S. Patent 4,353,888.

The following documents also teach immobilization of eukaryotic

cells in biocompatible gels of alginate, agarose, polyacrylamide, etc.

Nilsson, K. and Mosbach, K. in Preparation of Immobilized Animal Cells, FEBS Letters, 145-150 (1980) describe the entrapment of animal cells in several different types of gels.

Nilsson, K.,et al., in Entrapment of Animal Cells for the Production of Biomolecules such as Monoclonal Antibodies, 5th General Meeting of ESCAT, Copenhagen, Denmark, 1982, Develop. Biol. Standard, 55:155-161 (1984) and Nilsson, K., et al., in Entrapment of Animal Cells for Production of Monoclonal Antibodies and Other Biomolecules, Nature, 629-630 (1983) describe systems suitable for producing biomolecules from entrapped cells.

Problems related to cell loss from immobilized cell systems are addressed in several documents.

Cheetham, P.S.J., et al., in Physical Studies on Cell Immobilization Using Calcium Alginate Gels, Biotechnology and Bioengineering, 2155-2168 (1979) describes the use of calcium alginate gel pellets as a cell immobilization support and the undesired leakage of the cells from the gel especially when the entrapped cells are allowed to continue proliferating.

Casson, D. and Emery, A.N., in On the Elimination of Artefactual Effects in Assessing the Structure of Calcium Alginate Cell Immobilization Gels, Enzyme Microbial Technology, 102-106 (1987) describe how continued growth of cells entrapped within hydrogel beads leads to loss of cells into the nutrient medium, reducing the advantage of using immobilized cells.

Lim, F. and Moss, R.D., in Microencapsulation of Living Cells and Tissues, Journal of Pharmaceutical Sciences, 351-354 (1981) describe initially entrapping cells in alginate gels and then forming a membrane of another material on the gel surface in order to retain the growing cells within the gel bead and prevent their loss into the surrounding medium.

Rupp, R.G., in Use of Cellular Microencapsulation in Large-Scale Production of Monoclonal Antibodies, 19-38 (1985) describes maintaining a modified cytostatic culture of hybridoma cell by controlling rates of medium exchange and oxygenation, and the limitation of microencapsulation systems to batch production and harvest processes since the monoclonal antibody product is retained by the same molecu-

lar capsule used to retain the growing cells.

Nilsson, K., et al., in Production of Monoclonal Antibodies by Agarose-Entrapped Hybridoma Cells, Methods in Enzymology, 352-360 (1986) describe production of monoclonal antibodies by entrapped hybridoma cells, and suggests that low temperatures or nutrient limitations be used to avoid problems of cell escape from the gel due to unregulated growth.

Several reviews document the pleiotropic effects of butyric acid or salts thereof on specific cells and systems.

Prasad, K.N. and Sinha, P.K., in Effect of Sodium Butyrate on Mammalian Cells in Culture: A Review, In Vitro, 125-132 (1976) describe results of culturing several types of cells in the presence of sodium butyrate. The specific changes vary from cell to cell and the mechanism of action is not understood.

Kruh, J., in Effects of Sodium Butyrate, A New Pharmacological Agent, on Cells in Culture, Molecular and Cellular Biochemistry, 65-82 (1982) reviews the literature then available. At that time, known effects of butyric acid on cells included morphological changes, arrest of cell proliferation, induction of proteins, inhibition of cell differentiation, reversion of transformed characteristics of cells to normal morphological and biochemical pattern, increase in interferon antiviral efficiency, and induction of integrated viruses. The effects of butyrate were unpredictable; not all effects were seen in all cell lines and some modifications varied from one cell type to another without a reliable pattern.

Inhibition of cell growth by butyrate is described in several reports.

Hagopian, H.K., et al., in Effect of n-Butyrate on DNA Synthesis in Chick Fibroblasts and HeLa Cells, Cell, 855-860 (1977) describe the inhibition of cell proliferation and DNA synthesis of suspended cells cultured in the presence of sodium butyrate.

Leavitt, J., et al., in Butyric Acid Suppression of the In Vitro Neoplastic State of Syrian Hamster Cells, Nature, 262-265 (1978) describe changes in proliferation, morphology, anchorage-independent growth and fibrinolytic activity caused by butyric acid in three cells lines. The effects varied between one cell line and two related clones.

Leibovitch, M-P. and Kruh, J., in Effect of Sodium Butyrate on

Myoblast Growth and Differentiation, Biochemical and Biophysical Research Communications, 896-903 (1979) describe the inhibition of cell growth and differentiation in suspended myoblasts. These workers concluded the effect of butyrate strongly varies from one type of cell to another.

Other papers describing effects of butyric acid on cellular metabolism are Kamech, N., et al., in Butyrate Converts Rat 3T3 Fibroblasts into Giant Cells, Experimental Cell Research, 326-334 (1986); and Wintersberger, E., et al., in Butyrate Inhibits Mouse Fibroblasts at a Control Point in the G1 Phase, Journal of Cellular Biochemistry, 239-247 (1983).

The effects of butyrate treatment on the expression of specific endogenous gene products are discussed in the following documents.

D'Anna, J.A., et al., in Amino Acid Analysis and Cell Cycle Dependant Phosphorylation of an H1-like, Butyrate-Enhanced Protein (BEP; H1°; IP_{25}) from Chinese Hamster Cells, Biochemistry, 4331-4341 (1980) describe the isolation and sequencing of an intracellular protein produced by suspension cultures of CHO cells in response to the presence of butyrate.

Ginder, G., et al., in Activation of a Chicken Embryonic Globin Gene in Adult Erythroid Cells by 5-Azacytidine and Sodium Butyrate, Proc. Natl. Acad. Sci. USA, 3954-3958 (1984) describe the butyrate-induced activation of a gene which is normally not expressed in the cell line studied.

Polack, A., et al., in Truncation Does Not Abrogate Transcriptional Downregulation of the C-myc Gene by Sodium Butyrate in Burkitt's Lymphoma Cells, EMBO Journal, 2959-2964 (1987) document specific repression of the expression of endogenous immunoglobulin and oncogene products by addition of butyrate to culture medium.

Attempts to specifically modulate expression of genes in genetically engineered cells with butyrate have been described.

Gorman, C., et al. in Expression of Recombinant Plasmids in Mammalian Cells is Enhanced by Sodium Butyrate, Nucleic Acids Research, 7631-7648 (1983) describe the effect of sodium butyrate during transfection of mammalian cells, including CHO cells, to enhance protein expression. The study only monitored expression and was limited to three plasmids. The authors concluded the effect of butyrate on mammalian chromosomes is not uniform, that there are

regions on genes responsive to butyrate while other regions are not responsive, and that butyrate must be present during the initial transfection for there to be a subsequent induction of the genetically engineered gene upon later exposure of the transfected cells to butyrate.

In contrast, Yuan, Z-A., et al., in Effect of Butyrate on the Expression of Microinjected or Transfected Genes, Journal of Biological Chemistry, 3778-3783 (1985) report data suggesting inhibition of transfected gene expression at both the RNA level and at the protein translational level due to the presence of sodium butyrate. The dosing protocols were somewhat different than those used by Gorman, et al.

International Application Number PCT/GB87/00173, Production of Proteins by Cell Culture, published 24 September 1987, discloses increased yields of recombinant proteins and immunoglobulins when cells are cultured in the presence of sodium butyrate when the concentration of sodium butyrate used does not substantially decrease cell growth rate or significantly reduce cell viability.

Summary of the Invention

This invention provides a process for producing secreted proteins by cells cultured in a changeable growth medium, the improvement characterized by adding an effective amount of butyric acid or salt thereof to said growth medium. This process is particularly effective when the clone is immobilized on a microcarrier or within a permeable or porous gel bead. The use of immobilized cells treated with butyric acid or salts thereof allows the medium to be removed and replenished in either a batch or continuous manner without loss of cell viability or loss of cells to the surrounding medium. The invention is particularly useful for the production of monoclonal antibodies from hybridoma cells, and human renin or tissue plasminogen activator and analogs thereof from genetically engineered eukaryote cells.

Detailed Description of the Invention

The term effective amount means an amount of butyric acid or a sat thereof sufficient to modulate cell prolifieration and /or enhance gene expression levels without being toxic to the cell. The preferred amount is from about 0.1 to 10.0 mM.

In general, eukaryote cells secreting proteins are prepared by

the following general procedures which are known in the art. Cells are aseptically transferred from a frozen ampule to a flask containing nutrient medium. Once the cells have been transferred, the rectangular plastic flask is placed horizontally in an incubator at 37°C under a controlled gaseous environment and the cells are allowed to multiply. Suspension culture cells will multiply throughout the medium but generally settle to the bottom of the flask, whereas attachment dependent cells will require adherence to the submerged surface of the flask before growth occurs. The pH of the medium in the flask is buffered and phenol red, a pH indicator, is present to alert the investigator of adverse pH conditions. When the color of the medium indicates an adverse pH condition, the medium is replaced. After cell density reaches confluency on the surface of the flask the cells can be transferred to a larger flask. Suspension cells are released from the surface into solution by scraping with a spatula whereas attachment dependent cells are released from the surface by trypsinization.

Cells from T25 flasks, 25 ml, are inoculated in T150 flasks, 150 ml, with 75 ml of medium. Cells at this scale are treated similarly to the cells at the T25 scale. Cellular morphology, number and viability are determined microscopically. When the cell density reaches confluency, the cells can be either frozen for future use as a consistent supply of working stock preserving the original growth and production characteristics, or used as inoculum for the next increase in scale.

Cells that grow well in suspension are inoculated from T150 flasks into 1000 ml capacity spinner flasks. These flasks either utilize stirring bars, paddles, or a pendulum design to gently mix and aerate the cells without injury. The target cell concentration in the spinner just after inoculation is about $5x10^4$ to $1x10^5$ cells/ml. The minimum usable culture requires that at least a yield of $2.5x10^7$ cells are required from T150 flasks. The progress of a spinner is followed daily by viable counts. Once maximum cell density is achieved, spinners can be maintained at an inoculation density for the 10-liter scale by removing a portion of the old medium and cells in the spinner and replacing the volume removed with fresh medium.

Alternatively, attachment dependent cells can be cultured in

roller bottles. Attached cells from T150 flasks are freed from the surface by trypsinization and are inoculated into roller bottles. These plastic bottles have a total volume of approximately 2 liters but are used with only 500 ml of medium and are placed side down on a roller platform and slowly rotated around their axes. During this rotation attached cells will go through cyclic periods where during one portion of the cycle the attached cells will be submerged in the medium gaining needed nutrients and during the other portion of the cycle they will be above the liquid level with only a thin film of media adhering to them, thus allowing them to readily obtain oxygen for respiration. The cells are ready for inoculation into 10-liter vessels when the surface density approaches about $1x10^5$ to $2x10^5$ cells/cm^2. The cells from 2 to 4 roller bottles will yield a cell concentration after inoculation into a 10-liter vessel of about $2x10^4$ to $8x10^4$ cells/ml. The cell concentration in rollers is monitored visually by viewing the cells directly through the roller bottle with an inverted stage microscope. Cell densities starting at $1x10^4$ cells/cm^2 will in general require at least one feeding to achieve confluency.

Once cells reach an adequate inoculation density in spinner or roller bottles, they are introduced into 16-liter cell culture vessels. More specific details are found in Freshney, Culture of Animal Cells: A Manual of Basic Techniques (1983).

The cell lines generated by the outlined methods above are entrapped in alginate gels by known methods and cultured in medium which contains butyric acid. The medium can be changed in a cut-and-feed process in which all or nearly all the medium is removed from the cells and replaced in a batch type process or the medium can be changed in a continuous process in which new medium is continually added as old medium is removed. In either a cut-and-feed or continuous process one skilled in the art could change the make up of the medium to achieve maximum production of a particularly desired protein.

United States Patent Application Serial Number 07/023,491 filed 28 January 1987 teaches clones which secrete tissue plasminogen activator and analogs thereof and is herein incorporated by reference. The disclosure given in the above application's specification on page 6, line 23 through page 43, line 27 and the supporting charts

and tables on pages 44 through 83 contain specific procedural details.

The following examples describe how to perform the process of this invention. Those skilled in the art will promptly recognize appropriate variations from the described procedures which are included within the scope of this invention.

Example 1

Hybridoma 3D11 cells which secrete monoclonal antibody to a pseudorabies virus glycoprotein, as described in PCT Patent Application WO 8704075, are grown in a mixture of Dulbecco's Modified Eagles medium and Ham's F12 (DME/F12) containing 2% to 10% fetal bovine serum. The DME/F12 medium is a 50% mixture of each medium with a sodium bicarbonate concentration of 2.2 g/l. Cell density of stock cultures is maintained between $5X10^4$ to $1X10^6$ cells/ml in a 100 ml spinner flask culture.

The hybridoma cells are washed by centrifugation and resuspended in growth medium to $1X10^5$ cells/ml. Duplicate 100 ml spinner flask cultures are established at 37°C in the presence or absence of 0.5 mM butyrate. Cell counts are performed on aliquots removed at regular time intervals on a hemacytometer. Viability is determined by exclusion of Trypan blue dye. Antibody titers in the aliquots are determined by ELISA using goat-anti-mouse immunoglobulin antiserum. Results are given in Table 1.

Example 2

Chinese hamster ovary cells genetically engineered to express human prorenin from a recombinant, chromosomally incorporated chimeric gene under the control of an SV40 viral gene promotor are described in Poorman, R.A., et al., in "Isolation and Characterization of Native Numan Renin Derived from Chinese Hamster Ovary Cells" in Proteins: Structure, Function and Genetics, 134-145 (1986). Clone 100-6 cells are derived from a pool of CHO-renin cells adapted to growth in 100 nM methotrexate. They are maintained in MCDB-301 medium supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 nM) and fetal bovine serum (2%) as a monolayer in T-flasks or Petri plates. All cultures are maintained in a 37°C incubator with an 8% CO_2, 95% relative humidity atmosphere. Secreted prorenin is assayed as described by Poorman, et al.

The effect of butyrate on prorenin secretion from 100-6 cells is

shown in Table 2. CHO renin cell line 100-6 is grown in 25 cm^2 T-flasks. At confluence, the cells are washed and the medium replaced with fresh medium containing the indicated concentration of butyrate. After 48 hours the medium is removed, clarified by centrifugation and assayed for secreted prorenin.

Example 3

Murine C127 cells genetically engineered to express human prorenin from an extrachromosomal, recombinant bovine papillomavirus expression vector are described in Evans, D.B., et al., in "Isolation and Characterization of Human Prorenin Secreted from Murine Cells Transformed with a Bovine Papillomavirus-Preprorenin Expression Vector," Bio/Technology, 705-709 (1987). These cells are grown in DME/F12 medium supplemented with 10% fetal bovine serum on plastic cell cultureware. Once the cells attain confluence, growth medium is removed, the cells are washed with serum-free medium one time, and the cells are incubated with 10 ml/10 cm^2 surface area of serum-free DME/F12 medium $\pm$ butyrate (3 mM). Medium is changed daily (or as required by the nutritional requirements of the cells). Secreted prorenin is quantitated as described by Evans, et al. The data in Table 3 show butyrate treatment enhances expression driven by the extrachromosomal expression vector.

Example 4

$CHO_{12\text{-}500}$ cells, as described in European Patent Application 0-173-552, are grown in a mixture of Dulbeccos Modified Eagles medium and Ham's F12 medium (DME/F12) containing 7.5% fetal bovine serum (FBS) plus 500 nM methotrexate (MTX) in T-flasks and roller bottles. The DME/F12 medium is a 50% mixture of each medium with a sodium bicarbonate concentration of 2.2 g/l.

Gelatin microcarriers (Gelibeads^{TM}) are used at a concentration of 5 g/l in the 10 liter run. The procedures for their use are described below. Eight roller bottles are used to inoculate the 10 liter culture. This inoculum size has provided a good cell-to-microcarrier inoculation ratio for rapid growth and attainment of high cell concentrations.

A 10 liter New Brunswick Scientific Microgen^{R} culture vessel with a Cell Lift^{R} Impellor is used. Temperature, pH, dissolved oxygen and rpm are monitored by IBM-PC and Keithley DAS units, and controlled by these units or by control boards built into the vessel.

The operation of this system is described below.

Agitation is maintained at 30 rpm from inoculation to 90 hours, and is gradually increased to 55 rpm by 160 hours, to 65 rpm by 216 hours and then reduced to 50 rpm after 403 hours of the run. Agitation is increased from 100 to 120 rpm for 10 to 15 seconds prior to obtaining a culture sample for assay after 112 hours into the run when the culture is well established on the microcarriers. The pH is maintained between 7.3 to 7.5 by computer-controlled addition of 1N NaOH (for low pH control), or addition of CO_2 (for high pH control) into the culture vessel's head space. Dissolved oxygen is controlled by sparging. The dissolved oxygen concentration is maintained at 50-100% of the air-saturated concentration. Air is supplied to the head space at all times (about 1 to 2 l/min). Temperature is maintained between 36.3 and 36.7°C.

The DME/F12 medium containing FBS is used during the first two growth periods (0 to 188 hours) of the 10 liter run. MTX is not present in the medium at the 10 liter stage. Medium with FBS concentrations ranging from 0.1% to 2.0% is used in all culture periods of the run after the second harvest. Media containing 0.1% and 2.0% serum are also supplemented with 1 mg/l human transferrin (Sigma), 80 ng/l ferrous sulfate ($FeSO_4$&.$7H_2O$), 1 mg/l aprotinin, and 1 mg/l phospholipid from a liposome preparation containing 15% phosphotidylcholine and 5% ethanolamine. Butyric acid is added to media containing less than 1% serum to a final concentration of 3 mM during culture periods 3, 4 and 6.

During a cut-and-feed cycle agitation is stopped and microcarriers with attached cells are allowed to settle to the bottom of the vessel over a 10 minute period. Microcarrier-free spent medium (harvest medium) is then removed from the vessel, leaving 1.3 liters of medium containing settled microcarriers with attached cells at the bottom of the vessel. Ten liters of fresh medium are added back to the vessel. This process is repeated every three days through harvest 4 at 332 hours of the run. The fifth culture period is increased to 5 days (332 to 454 hours). The sixth and final culture period is increased to 7 days. TPA production following this procedure is given in Table 4.

Example 5

European Patent Application EP 0231624 discloses human tissue

plasminogen activator (tPA) analogs with rearranged, deleted and/or additional sequences and their expression by rDNA techniques.

Exponentially growing dihydrofolate reductase-deficient Chinese hamster ovary (CHO) cells are trypsinized using a solution of 0.25% trypsin (no EDTA) in 1x PBS (GIBCO) 24 hours prior to transfection. Approximately $5x10^6$ cells are seeded into each T25 flask (all plasticware from Corning) to be transfected. Three hours prior to addition of the DNA precipitate cells are refed. Media for non-transfected CHO cells consist of Ham's F12 nutrient mixture supplemented with 10 mM HEPES, 100 units penicillin/ml, 100 μg/ml streptomycin and 10% heat inactivated fetal bovine serum (all from GIBCO). Cells for transfection are carried for no more than 10-15 passages, after which a new vial of cells is thawed.

Precipitates are formed as follows. One mcg of plasmid DNA from a pSV2dhfr-based expression vector using the cytomegalovirus immediate early promoter (CMV IE) and the bGH polyadenylation signal sequence in from 1-10 μl sterile TE (10 mM tris pH 8.0, 1 mM EDTA) and 31 μl of 2M CaCl) are added to 250 μl TE plus carrier DNA (10 mM tris pH 8.0, 1 mM EDTA, carrier DNA at 5 μg/ml) in a sterile plastic tube. This mixture is removed to a second tube by alternately drawing air, then DNA is mixed into a sterile pipette and the solution is bubbled slowly into 250 μl 2X HBS (2X HBS is 0.28M NaCl, 0.05M HEPES pH 7.1, 3 mM Na_2HPO_4; pH is adjusted to 7.1 with NaOH; filter sterilized, stored at 4°). Further mixing is by gentle aeration, accomplished by gently bubbling air into the solution via sterile pipette. The DNA precipitate is allowed to form for 30 minutes at room temperature after which it is added to the cells. The cells are incubated with the precipitate under a 5% CO_2 humidified atmosphere at 37°C for between 4-8 hours.

After the incubation period the medium containing the DNA precipitate is removed and 1.5 ml of a 15% glycerol/HBS solution (room temperature) is added to each flask. Cells are immediately placed at 37°C for one minute after which the glycerol is diluted with 5 ml Ham's F12 medium and quickly removed. Cells are washed 3 times, 5 ml/wash, with Ham's medium, and finally fed with 5 ml Ham's per T25 flask. The transfected cells are placed at 37°C for 48 hours after which they are trypsinized and passed to a T150 flask containing selective medium made from high glucose (4500 μg/L) Dulbecco's

Modified Eagle's Medium (DMEM plus glutamine) supplemented with 10% heat inactivated fetal bovine serum, 0.1 mM MEM nonessential amino acids, 10 mM HEPES, pH 7.3, 100 units penicillin/per ml and 100 μg/ml streptomycin. Stable transfectants appear as foci within 7-10 days after seeding into selective medium. The foci are trypsinized and seeded into a T75 flask in selective medium. Upon formation of a monolayer, cells are trypsinized for scale up and for storage.

Stable transfectants derived via transfection are subsequently butyrate induced after growing cells to approximately 90% confluency in T75 flasks. Spent medium is removed and cells are refed with fresh medium minus butyrate. Uninduced conditioned culture medium is harvested for assay 24 hours later. The same monolayers are then refed with fresh medium containing 5 mM butyrate. Induced conditioned culture medium is harvested 24 hours later and assayed for tPA activity.

Transfected gene copy number is further increased by gene amplification. Cells are adapted to growth in 100 nM and then 500 nM MTX according to Kaufman, et al., Mol. Cell. Biol., 5:1750 (1985). Pools of transfectants are seeded at about 80% confluency into T150 flasks in medium plus MTX. The medium is changed every 3 to 4 days. A successful adaptation is indicated by a die-off of the monolayer followed by development of surviving foci identical in appearance to that of a flask of successfully transfected cells in selective medium. Foci are allowed to grow out (about 7 days) before trypsinization and passaged without dilution. After establishment of a new monolayer in MTX, cells are assayed and/or used for tPA/tPA analog production. The results are given in Table 5.

The amplified cell lines 12/100-1 (tPA-cDNA) and 12/500-2 are grown to confluency in duplicate 100 mm Petri plates. At confluency, the growth medium is removed, the cell monolayers are washed 2 times with phosphate buffered saline and fed 10 ml of serum-free medium containing insulin, transferrin, and selenium. After 48 hours the medium is harvested and assayed for tPA by ELISA or by measuring activity inhibitable by the active site directed monoclonal antibody ESP-2 in a direct activity assay using the S-2288 chromogenic substrate. The results are given in Table 6.

Example 6

UG-4 cells are Chinese hamster ovary cells genetically engi-

neered to express the FK_2P analog of human tPA (which is a deletion mutant lacking the "growth factor" and first "Kringle" domains).

UG-4 cells can be maintained routinely as monolayers in growth medium containing 5% FBS.

The UG-4 cells are suspended by rinsing with a HEPES buffered saline (HBS) and a brief incubation with PET (1% polyvinylpyrolidone, .004% [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) and .02% trypsin in HBS). The cells are then diluted in cold culture medium, a sample is taken for a viable cell count, and the remaining cells are collected by centrifugation for 8 minutes at 50 xg. The cell pellet is resuspended at a concentration of $2X10^6$ cells/ml. One volume of cells is added to 19 volumes of 1.05% sodium alginate (Bellco Glass, Inc.) which is previously made isotonic by the addition of 0.26 g of NaCl per 100 ml of alginate solution.

After thorough mixing, the alginate cell suspension is expressed through a 20 g needle into a stream of sterile air. The resulting droplets become insoluble calcium-alginate beads when they enter a solution of 50 mM $CaCl_2$, 5 mM HEPES and 83.5 mM NaCl (pH 7.3). The final bead size is controlled by both the rate that the suspension is expressed through the needle and the flow rate of the sterile air and is determined empirically. The 50 mM $CaCl_2$ solution is 3-4x the volume of the alginate-cell suspension. Once the beads are formed, the calcium chloride solution is removed by aspiration. The beads are then washed 4x with isotonic HEPES saline (IHS) (150 mM NaCl, 2 mM HEPES, pH 7.3). A final wash is carried out using growth medium.

The beads are then transferred in serum-containing medium to the vessel being used for culturing the immobilized cells. A 150 ml bead volume of immobilized UG-4 is prepared for culture in a disposable airlift fermentor (ALF) (Cellift, Ventrex Laboratories, Inc.). The ALF culture is initiated with growth medium containing 2% FBS, 50 μg/ml gentamicin sulfate and 0.5% antifoam (Ventrex Laboratories). A volume of 380 ml of medium is combined in the ALF with 150 ml of beads. The medium and beads are constantly circulated by the airlift action with a 5% CO_2 in air gas feed. The culture temperature is maintained at 36°C by a thermostatically controlled heating jacket. During the first days of culture, fresh medium can be provided by batch feeding: the gas source is shut off causing the beads to settle, the spent medium is poured into a collection bottle, and

fresh medium is pumped in. As cell number increases, the ALF is converted to a perfusion mode. A perfusion pump system is used to pump fresh medium into the ALF and spent medium out. Beads are retained through the use of a wide withdrawal tube in which the beads settle at a faster rate than medium is withdrawn. The fresh medium feed bottle is maintained at 4°C by storage on ice, or in a refrigerated water bath. During perfusion culture, the cell environment can be modified by varying medium flow rates, serum levels, and by the addition of sodium butyrate as described below. A summary of tPA production for this example is given in Table 7.

During the first two weeks of culture the ALF medium requirement is determined based on glucose utilization and pH. On day 15, perfusion is started and the ALF is fed serum-free medium containing 1 μg/ml phosphatidylcholine liposomes. As the serum concentration in the ALF decreases with the constant perfusion of serum-free medium, the tPA productivity of the cells decreases (day 15-20). On day 26, serum is added back to the medium feed and also injected directly into the ALF at a concentration of 0.5%. In less than 24 hours the production of tPA increases from 0.23 μg/ml to 1.07 μg/ml. On day 30, butyric acid is added to the medium feed, and injected directly into ALF at a concentration of 3 mM. When the spent medium is analyzed for tPA content, the tPA level has increased from 1.5 μg/ml to 3.9 μg/ml. The butyrate is later increased to 10 mM on day 34. On day 37 the butyrate is removed from the medium feed; with continued perfusion the butyrate content decreases approximately 50% each day. The tPA content in the medium remains stable over several days, then slowly decreases to the level seen before butyrate induction. On day 51, 3 mM butyrate is reintroduced into the system. The tPA content of the medium again increases until the ALF culture is intentionally terminated on day 55, at which point the tPA level has reached 4.4 μg/ml.

TABLE 1

Time (hours)	Concentration of Antibody (μg/ml) With Butyrate	Concentration of Antibody (μg/ml) Without Butyrate	Cell Count (10^5 cells/ml) With Butyrate	Cell Count (10^5 cells/ml) Without Butyrate
0	0.15	0.1	0.8	0.6
15	0.42	0.4	1.3	1.3
24	0.56	0.4	1.6	2.2
39	1.0	0.7	2.1	4.9
48	--	1.0	2.6	5.9
72	2.9	2.0	3.5	10

TABLE 2

ProRenin Production

Butyrate (mM)	Prorenin μg/ml
0	5
0.1	6
0.5	7
1.0	7
5.0	8
10.0	10

TABLE 3

Butyrate Enhanced Secretion
From Murine C127-BVP-Renin Cells

	With Butyrate	Without Butyrate
Prorenin secreted	2.5 μg/ml	1.1 μg/ml

TABLE 4

tPA Production in Cell Line CHO_{12-500}

Harvest	Butyrate (mM)	Serum (%)	tPA (ng/10^6 cells/day)
1	0	7.5	3.3
2	0	7.5	3.6
3	3	0.9	17.0
4	3	0.9	5.0
5	3	2.1	22.0
6	3	0.1	9.0
7	0.5	2	4.3
8	0.5	2	5.6
9	0.5	2	2.6
10	0.5	2	3.3
11	3	0.3	9.0
12	3	0.3	3.3
13	3	0.3	1.0

TABLE 5

tPA analog	tPA activity (IU/10^6 cells/day) Without Butyrate	With Butyrate	Fold Induction
FK_2P	52	227	4.4
FK_1K_2P	36	116	3.2
FGK_1P	4	8	2.0
FGK_1K_2P	56	159	2.8
FGK_2K_1P	16	71	4.4
GK_1K_2P	2.4	6.7	2.8

TABLE 6

Cell Line	tPA activity (IU/10^6 cells/day) Without Butyrate	With Butyrate	Induction
12/100-1	1907	4451	2.3
12/500-2	1734	8901	5.1

TABLE 7

tPA Production

Day in Culture	tPA (mcg/ml)
15	1.5
20	0.23
25	0.23
30	1.25
35	3.0
40	2.3
45	1.9
50	1.8
55	4.4

CLAIMS

1. A process for producing proteins from eukaryotic cells cultured in a changeable growth media the improvement characterized by adding an effective amount of butyric acid or salt thereof to said growth medium.

2. A process according to claim 1 wherein said eukaryotic cells are hybridoma cells able to secrete monoclonal antibodies.

3. A process according to claim 1 wherein said eukaryotic cells are transformed cells genetically modified to express a protein from a recombinant gene.

4. A process according to claim 3 wherein said recombinant gene is incorporated into the transformed cell chromatin.

5. A process according to claim 3 wherein said recombinant gene is maintained in an extrachromosomal plasmid.

6. A process according to claim 4 wherein said recombinant gene's copy number has been increased by gene amplification.

7. A process according to claim 1 wherein said eukaryotic cells are immobilized.

8. A process according to claim 7 wherein said eukaryotic cells are immobilized on an inert attachment surface.

9. A process according to claim 7 wherein said eukaryotic cells are immobilized within an inert matrix.

10. A process according to claim 9 wherein said inert matrix is formed by an alginate gel.

11. A process according to claim 1 wherein the growth medium is changed in a cut-and-feed process.

12. A process according to claim 1 wherein the growth medium is changed in a continuous process.

13. A process according to claim 7 wherein said butyric acid is added to the growth medium after the cells have reached a production density.

14. A process according to claim 3 wherein said eukaryotic cell is a Chinese hamster ovary cell or a mouse C127 cell.

15. A process according to claim 3 wherein said protein is human tissue plasminogen activator, human prorenin, or analogs thereof.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/00033

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [8]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 5/02; C 12 N 11/10; A 61 K 37/02; C 12 N 1/38

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	WO, A, 87/05626 (CELL-TECH LTD) 24 September 1987 see page 12, line 1 - page 23, line 20 cited in the application	1-15
X	Hybridoma, volume 4, no. 1, 1985, C. Williams et al.: "Hybridomas increase antibody production when treated with butyric acid", page 63 see the whole article	1-15
X	Nucleic Acids Research, volume 11, no. 21, 1983, C.M. Gorman et al.: "Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate", pages 7631-7648 see page 7631, abstract	1-15
X	Chemical Abstracts, volume 97, no. 17, 25 October 1982, (Columbus, Ohio, US), ./.	1

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
31st March 1989	27 APR 1989
International Searching Authority: EUROPEAN PATENT OFFICE	Signature of Authorized Officer: P.C.G. VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	J. Shuttleworth et al.: "Protein synthesis in human lymphoblastoid cells (Namalwa) after treatment with butyrate and 5'-bromodeoxyuridine", see page 27, abstract 138196c, & Biochim. Biophys. Acta 1982, 698(1), 1-10	
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X	Chemical Abstracts, volume 108, no. 15, 11 April 1988, (Columbus, Ohio, US), S.J. Hong et al.: "Activation of protein expression in clonal glioma cells by sodium butyrate", see page 23, abstract 124145u, & Kao-hsiung I Hsueh K'o Hsueh Tsa Chih 1987, 3(11), 695-702	1
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X	Biological Abstracts, volume 71, no. 8, 1981, (Philadelphia, PA., US), P. Milhaud et al.: "Sedium butyrate affects expression of fibronectin on CHO cells: Specific increase in antibody-complement-mediated cyto-toxicity", see page 5486, abstract 52270, & J. Cell. Physiol. 104(2): 163-70, 1980	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8900033 SA 26508

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 18/04/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8705626	24-09-87	EP-A- 0239292	30-09-87
		AU-A- 7126487	09-10-87
		GB-A- 2196636	05-05-88
		JP-T- 63503273	02-12-88